Vol. 90, No. 3 June, 1946

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF ARBACIA PUNCTULATA. II. EFFECT OF THE MEDIUM ON RESPIRATION

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Introduction

The first section (Hayashi, 1945) of this investigation presented evidence for the existence of a factor in the seminal fluid of Arbacia which influences the duration of fertilizing capacity of the sperm of the same species. The experimental results indicated that the factor was adsorbed on the sperm surface, and subsequently was lost into the surrounding medium. Since Gray (1928a) has shown a relation between "mechanical crowding" of sperm and the length of metabolic life of sperm, the question at once arose as to whether the seminal fluid factor influenced the fertilizing power of sperm through an effect on the metabolism of sperm. Consequently, a series of experiments was done investigating the effects of seminal fluid on the respiratory activity of sperm.

Materials and Methods

The microrespirometer used for these experiments was a compensating type, a modification of Krogh's respirometer.[1] It consisted of two similar vessels of conventional design connected through a common U-shaped manometer. To remove the carbon dioxide, filter paper wet with 20 per cent KOH was placed in the manometer vessels out of contact with the respiring cells. The manometer vessels were immersed in a constant-temperature bath maintained steadily at 25° C. The shaker, upon which the microrespirometers were mounted, moved to and fro in a straight line a distance of 15 centimeters. A steady rate of 40 cycles per minute thoroughly agitated the respiring sperm suspensions. The sensitivity of the respirometer was such that a respiratory rate of one mm.^{3} of oxygen consumed per hour could be detected in ten-minute readings.

Two points of difficulty were encountered in the course of these experiments. First, capillary action prevented the easy transfer of any liquids from the side-arm into the respirometer vessel. Second, the effect of dilution on the respiration of sperm was found to be very rapid, and therefore difficult to measure. To overcome these difficulties, a measured amount of packed sperm was placed on the glass wall

[1] Kindly lent by Dr. Titus C. Evans, formerly of Iowa State University.

of the manometer vessel above the point reached by a given amount of liquid in the vessel, when this liquid was agitated by the movements of the shaker. The packed sperm remained in place by their own adhesive action. When the time came to dilute the sperm in the medium to be studied, the adhered sperm on the side of the vessel were washed down into the liquid by a slightly greater agitation of the manometer vessel.

This technique overcame the first of the above-mentioned difficulties satisfactorily, but only partly the second. To wash down and to disperse completely the sperm cells in the medium required time in the order of minutes. Since, during this period, changing numbers of cells were being affected by the medium, neither the total respiration nor the total respiratory rate could be accurately measured. This effect was minimized by manipulation of the vessels and by extrapolation of the respiration curve.

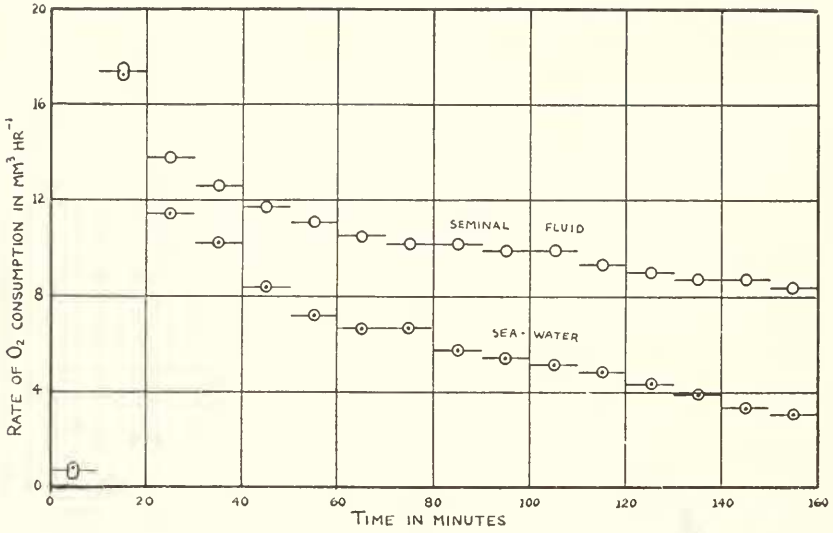

FIGURE 1. Effect of seminal fluid, sea water on respiratory rate of sperm.

The pH of the medium in the experiments here presented was not checked after each run, although it was known before the run, and has been published elsewhere (Hayashi, 1945). In later, similar experiments (to be published) the pH was adjusted to equality and checked before and after each run. In the time of the run, no change in pH occurred, and the results did not differ from those presented.

It was noted that the sperm in all the respiration experiments, irrespective of the medium used, and in the concentrations of these experiments, were dead at the end of five hours. A sharp rise in the rate of oxygen consumption occurs at about this time, due, probably, to disintegration of the sperm cells caused by the constant shaking of the manometers. This same effect was noted by Gray (1928b, p. 350). This effect afforded no trouble in the interpretation of the results, however, for it occurred uniformly in all sperm suspensions, and the pertinent data were obtained before the end of the five-hour period.

Experiments and Results

For the first experiments, the respiration of sperm in seminal fluid was compared with the respiration of an equal amount of sperm in sea water. Each suspension contained 0.00155 cc. of packed sperm per cc. of medium. The contrasting changes in the respiratory rates of the two suspensions are shown in Figure 1. Using the same data, the total oxygen consumed was plotted as a function of time, to produce the curves of Figure 2.

The experiment showed that the seminal fluid did not affect the sharp increase in respiratory activity immediately following the dilution of the sperm. The main effect of the seminal fluid was to regulate, or delay, the sharp drop in respiratory rate shown by the sperm in sea water. Figure 1 also showed that the total amount of energy expended by equal amounts of sperm in the same length of time was greater when the sperm were suspended in seminal fluid.

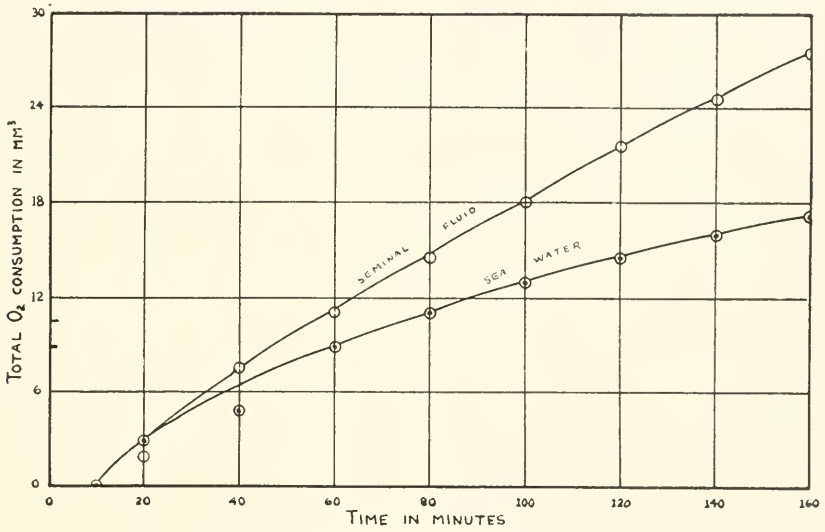

Figure 2. Total oxygen consumption as a function of time for sperm in seminal fluid and sea water.

As a further check on the effect of dilution on the respiration of sperm, one of Gray's (1928a) experiments was repeated, with both seminal fluid and sea water. A measured quantity of packed sperm (0.00155 cc.) was placed on the wall of the respiration chamber, and 1.0 cc. of medium was placed on the bottom of the vessel. In addition 0.5 cc. of medium was placed in the side arm, and this amount of medium was "dumped" over into the respiration chamber at an appropriate time. The results of the experiment are shown in Figure 3.

The results confirmed Gray, but compared with his results, the degree of rise in the respiratory rate at the second dilution was less. When the sperm concentrations used by Gray were compared with those used here, it was seen that the present sperm suspensions were far more dilute. Therefore, the results were taken to mean that the concentration of sperm in this experiment was near the

upper limit to obtain the maximum burst of energy at first dilution from the amount of sperm used. In other words, greater original dilution of the packed sperm would not result in very much more initial activity.

This interpretation was partly confirmed by the following experiment. Lillie (1913) had found that egg water stimulated sperm to greater activity, and Gray (1928c) concluded from his experiments that egg water stimulated sperm to greater respiratory activity. However, Gray had used egg water as the first and only diluent for the sperm. In the following experiment, by contrast, the egg water was used as a diluent after the initial burst of activity induced by the original dilution of the medium.

The same procedure as the preceding experiment was employed. Instead of the respective media in the side arms, however, egg water was used, and this was

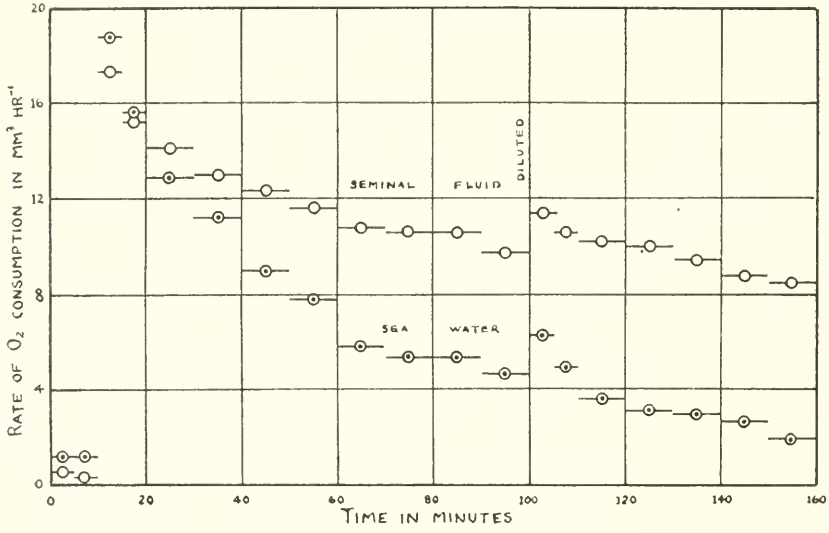

Figure 3. Effect of dilution by respective media on sperm in sea water and seminal fluid.

"dumped" in at the appropriate time. The results are given in Figure 4. Study of Figure 4 showed that, after the original burst of activity, egg water, far from stimulating the sperm, seemed to inhibit them. The effect was probably due to the agglutination of the sperm, and seemed to overcome the slight stimulation due to dilution shown in Figure 3.

Discussion

Analysis of the effect of the seminal fluid on sperm respiration

The respiratory rate of sperm in seminal fluid is compared with that of sperm in sea water in Figure 1. The seminal fluid does not seem to influence the initial steep rise in activity due to dilution. There was some variation shown in this first burst of respiration, but a check of all the runs made showed that the variations were not significant. In some cases the sperm in seminal fluid showed more intense

activity at dilution; in others, the sperm in sea water were more active at dilution. The variations were caused probably by differences in the rate at which the sperm were washed from the sides of the respiratory vessels.

The effect of the seminal fluid is on the "senescent period" following the initial respiratory rise. The seminal fluid seems to prevent the rapid decline of the metabolic rate evident in the sea-water control. The effect of the seminal fluid on the respiration of sperm thus parallels the effect of seminal fluid on the fertilizing power of sperm. In both cases, the seminal fluid maintains the sperm at a high functional level for a longer time than does sea water. A comparison of the areas under the curves shows that sperm in seminal fluid expend more energy than an equal amount of sperm in sea water during the course of their active lives. There are two possible explanations for these results. It is possible that the sperm in seminal fluid utilized

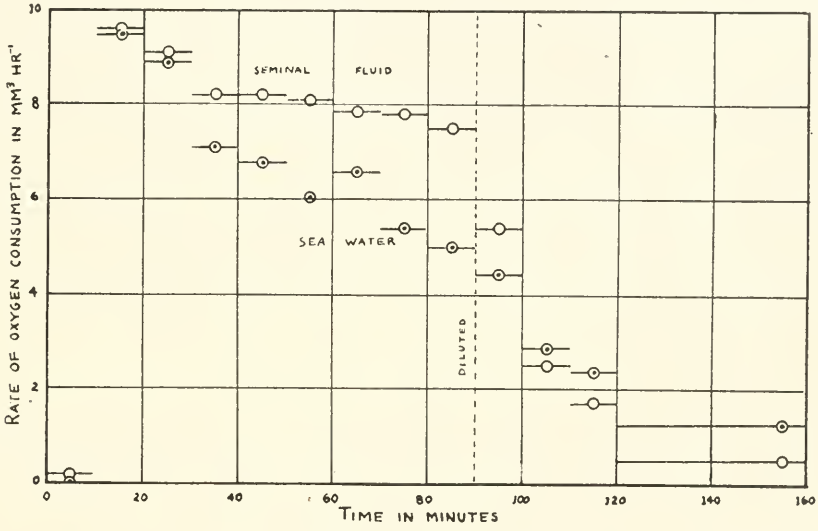

FIGURE 4. Effect of dilution by egg water on sperm in sea water and seminal fluid.

their internal store of fuel more completely than the sperm in sea water. The second possibility is that the seminal fluid is serving as a source of fuel for the sperm. A clue to these possibilities was sought by utilization of some of Gray's analytical methods.

Gray (1928b) originally advanced the view that the sperm cell was a tiny combustion engine with a limited supply of fuel. From his original assumption, Gray derived the equation

$$\text{Activity} = \frac{dx}{dt} = k(a - x) \qquad \text{(Equation 1)}$$

which, integrated, gave

$$k = \frac{1}{t} ln_e \frac{a}{a - x}. \qquad \text{(Equation 1a)}$$

In this equation, x represented the amount of fuel used up in time, t; a, the initial, total amount of fuel in the sperm cell; and k, the activity produced by each unit of fuel consumed. Gray found that this "law of exponential decay of a homogeneous population" did not fit the experimental results. He, therefore, expanded the equation with a consideration of a heterogeneous population, and derived, as one form of the equation,

$$x = O_2\left(\frac{t}{\alpha + t}\right), \qquad \text{(Equation 2)}$$

in which O_2 represented the total oxygen consumed during active life; α, another constant; and x and t, possessing the same meanings as before. Gray found that this equation could be made to fit the experimental results, and concluded that the sperm population was heterogeneous in a special way, and that its activity decreased because of the depletion of an internal supply of energy. The notion of heterogeneity was used as a basis for a later, more detailed mathematical analysis (Gray, 1930).

The above methods were applied to the results of a number of experiments of this investigation. At two different values of time t in a single run, experimental values of the total oxygen consumption were arbitrarily selected, and the values of the constants k, a, O_2, and α in equations 1a and 2 were calculated. The values of the constants thus derived were then substituted back in the equations and the theoretical values of x for the entire run calculated and compared to the experimental values. The results showed that, with the proper selection of points, both equations 1a and 2 could be made to fit the experimental data rather closely, but other points could be selected to demonstrate that neither of the equations fit the experimental data. The latter case, in a typical experiment, is shown in Table I.

TABLE I

Comparison of the experimental and theoretical values of the total oxygen consumption of sperm in sea water and seminal fluid. Theoretical values calculated from equation 1a and equation 2

Time (minutes)	Total oxygen consumed in mm.³					
	Sea water			Seminal fluid		
	Exp.	$k = .01275$ $a = 12.24$	$O_2 = 15.25$ $\alpha = 71.15$	Exp.	$k = .00876$ $a = 16.31$	$O_2 = 22.54$ $\alpha = 135.7$
0	0.00	0.00	0.00	0.00	0.00	0.00
10	1.05	1.45	1.87	0.95	1.36	1.55
20	2.07	2.75	3.32	1.99	2.62	2.90
30	3.09	3.89	4.50	3.08	3.77	4.08
40	4.06	4.89	5.46	4.06	4.83	5.13
50	4.96	5.77	6.26	5.06	5.78	6.07
60	5.84	6.55	6.94	6.04	6.66	6.91
70	6.68	7.23	7.53	6.97	7.47	7.67
90	8.08	8.36	8.49	8.60	8.90	8.99
100	8.70	8.86	8.88	9.37	9.52	9.56
110	9.23	(9.23)	(9.23)	10.09	(10.09)	(10.09)
210	11.41	11.40	11.37	13.80	13.72	13.69
220	11.50	(11.50)	(11.50)	13.94	(13.94)	(13.94)
255	11.76	11.76	11.90	14.31	14.56	14.71

It may be noted that the points selected are those covering the end of the run, and the data of this portion fit the experimental values rather closely, whereas wide variation is evident in the early portions of the run. Points selected near the beginning of the run, in contrast, would show a fit in that portion of the run, and variation at the end. Intermediate points, of course, would show an intermediate fit.

From such analyses, these conclusions may be drawn. (1) There is no need to assume heterogeneity of the sperm population whether in sea water or seminal fluid. (2) The simple "law of exponential decay" is not adequate to explain sperm activity; the decrease in activity of the sperm cells with time is not due to the exhaustion of an internal supply of fuel alone, whether the sperm cells are in sea water or seminal fluid.

Since sperm cells in sea water show a decline in activity whose controlling factor is not the exhaustion of their internal source of fuel, it is possible that even at the end of active life in this medium, the sperm cells contain unused, potential energy. The utilization of this unused energy by the sperm in seminal fluid would explain the increased oxygen consumption of the sperm cells in seminal fluid. There is, however, the possibility that the sperm in sea water reach the end of active life because of starving *plus* other factors. The question of nutrition by seminal fluid, therefore, is unsettled by the above analyses, although strong possibilities are afforded.

Gray (1928b) analyzed the senescence of spermatozoa from still other considerations. He assumed that the cause of the death of the spermatozoon was the accumulation of products of metabolic activity inside the sperm cell. This would render inactive part of the active system originally present in the cell.

$$\text{Active system} + \text{product of activity} \rightleftarrows \text{inactive system.}$$

Based on this concept Gray derived an equation which he called the "theory of autointoxication."

$$x = \sqrt{\frac{2Ka}{b}}\,\sqrt{t}. \qquad \text{(Equation 3)}$$

Here x, a, and t designated the same quantities as before, and K and b were constants. Equation 3, based primarily on the assumption of a homogeneous population, showed that the amount of fuel, x, consumed in time t was directly proportional to the square root of t. Therefore, the total oxygen consumed should show a straight-line relationship to the square root of the age of the active suspension. Gray found that this relationship did not hold for sperm in sea water, for toward the end of active life the points fell below the expected values. He concluded that part of the active system was not only being inactivated, but also being irreversibly destroyed.

Equation 3 was applied to the experimental figures of the present investigation. When the total oxygen consumption of the sperm in sea water is plotted against the square root of the time, the results confirm Gray's findings, for, toward the end of the run, the points fall below the expected values. (The variation from the straight-line relationship at the beginning of the run is due to the time requird for the cells to be completely dispersed in the medium.) For the sperm in sea water, therefore, an irreversible destruction of part of the active metabolic system is taking place to-

ward the end of the run (Fig. 5). In contrast, the straight-line relationship is followed for a much longer time in seminal fluid in an equivalent run (Fig. 6). In some of the experiments, a departure from the straight-line relationship occurred in the seminal fluid, but in all cases, this departure occurred very much later from the beginning of active life. In the seminal fluid, therefore, the irreversible destruction of the active system is much delayed, as compared to the case of the sperm in sea water. The decay of activity of the sperm in seminal fluid is apparently due, at least for a great part, to a process of autointoxication.

The difference between the metabolism of sperm in sea water and in seminal fluid apparently lies in the fact that some sort of metabolic system is kept intact for a longer time when the sperm cells are suspended in seminal fluid. The sperm and a factor in the seminal fluid, therefore, constitute a closed system. In sea water,

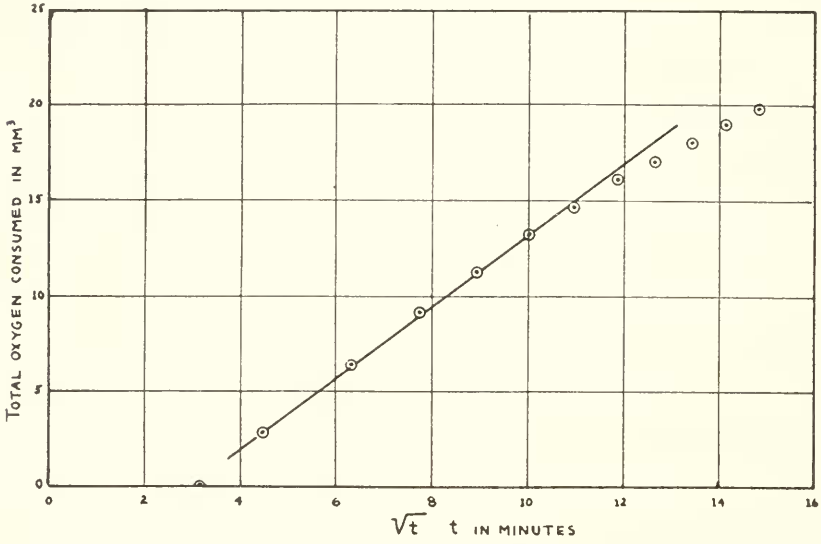

FIGURE 5. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in sea water.

part of this closed system is destroyed quite early during the active life of the sperm.

The above findings, supported by the knowledge that seminal fluid contains no reducing sugars (Hayashi, 1945), lends further support to the idea that the effect of seminal fluid on sperm respiration is not due to the replenishment of a store of energy, but rather to the maintenance of a metabolic system. Other experiments (unpublished data) measuring the R.Q. of sperm cells both in sea water and seminal fluid show a characteristic carbohydrate metabolism for these cells, further supporting the contention that the seminal fluid does not provide added nutrients for the sperm cells. It may be concluded that, although there is the possibility that sperm can be nourished experimentally, it is most important for future experimentation that the system, of which the sperm cells are only a part, be kept intact. Workers who have diluted sperm cells in sea water have diluted not only cells, but, also, system.

Both the fertilizing power (Hayashi, 1945) and the respiratory activity of sperm are maintained by a common factor, the seminal fluid. As a first possibility, the role of the seminal fluid factor may be considered fundamental to the respiratory activity only, the fall in fertilizing power being a manifestation of the loss of activity of the sperm cells. In other words, the fall in fertilizing power is only a secondary effect in relation to the seminal fluid factor. The second possibility is that the seminal fluid plays a part directly in both fertilization and respiration. Lack of data does not permit the choice of these possibilities. However, the fact that a seminal fluid factor influences sperm respiration is implicit in both possibilities.

A mechanism, tentatively proposed earlier as the wearing off of a protein substance from the surface of the sperm cell, was adequate to explain the loss of fertilizing power of sperm in sea water. The same mechanism is also applicable to the

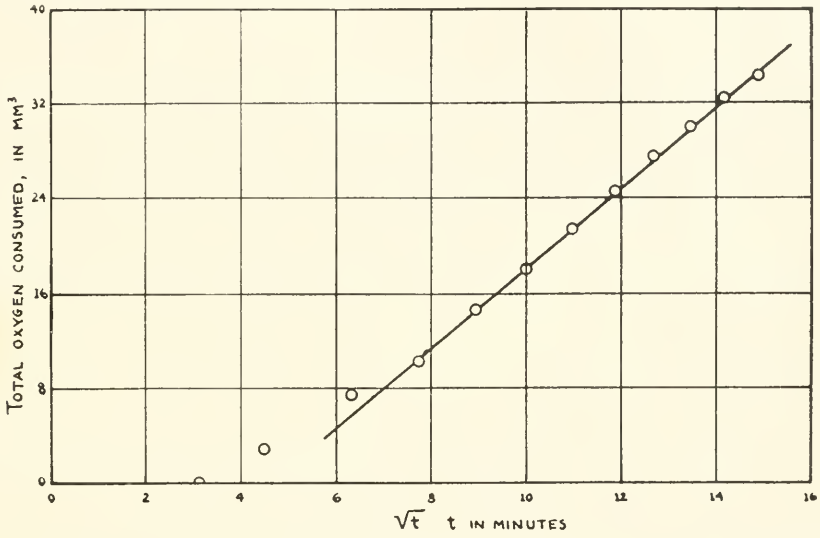

FIGURE 6. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in seminal fluid.

results of respiration studies, if the assumption be made, that the removal of each molecule of protein substance from the surface of the sperm cell releases to the sperm cell a finite amount of the total internal energy. The rate of sperm respiration thus would be controlled by the rate of removal of substance from the sperm surface, which is in turn controlled by other factors. The principal factor affecting the rate of removal of a sperm-surface-substance is the "mechanical crowding" factor studied by Gray (1928a), who found that sperm cells exhibited a burst of activity when diluted. This was confirmed in the present study (Figs. 1, 3, 4). According to the mechanism proposed, the rate of loss of sperm-surface-substance is inhibited when the cells are in a crowded condition, but when relatively far apart, the removal of surface-substance is enhanced.

The removal of sperm substance from the cell surface presumably constitutes the irreversible destruction of part of the active system noted in Fig. 5. In seminal fluid,

however, the replacement of the surface-substance would delay the onset of this irreversible destruction (Fig. 6), the senescence of the sperm cells being conditioned by internal autointoxication, and perhaps also by depletion of the internal energy. It would seem that for the sperm cells in sea water, the destruction of part of the system, in addition to autointoxication and probable depletion of fuel, controls the fall in activity.

It is understood that the mechanism as outlined is tentative at best, but it serves as an explanation of the facts so far known. The facts fit well with the earlier results of the effect of seminal fluid on the fertilizing capacity of sperm. It may be concluded that possibly a common mechanism underlies the effect of seminal fluid on the fertilizing function and the respiration of sperm. The relationship between the surface-substance and the respiratory mechanism of the sperm cell was not investigated. The action of the surface-substance while attached to the sperm cell is possibly enzymatic.

Effect of egg water on sperm respiration

Egg water does not stimulate the respiration of sperm cells but seems to condition a sharp drop in the respiratory rate (Fig. 4). When egg water was added to sperm suspended in sea water and, also, to sperm suspended in seminal fluid, the effect was a sharp decrease in the respiratory rate. This decrease of metabolic activity upon dilution with egg water is in contrast to the effect of dilution with sea water and seminal fluid shown in Figure 3. The increase in respiratory rate brought about by dilution with sea water and seminal fluid shows that even at the dilution used, "mechanical crowding" was still apparently a factor inhibiting the respiration of spermatozoa. Dilution with egg water also relieved the "mechanical crowding," but the agglutinating effect of egg water apparently overcame the effect of dilution so that the respiratory activity decreased.

The stimulation of metabolism by egg water noted by Gray (1928c) and Carter (1930) is not confirmed in these experiments. However, it may be recalled that Carter (1931) had found no stimulation of ripe sperm by egg water. In addition, the egg water of the present investigation was added, not to undiluted sperm, but to sperm that had already been activated by an initial dilution. Therefore, it is possible either that egg water did not affect the respiration of sperm at all, or that the difference in the time of addition of egg water was the cause of the disparity of the results of Gray's experiments and the results of the present investigation.

Summary

1. Seminal fluid has the property of delaying the fall of respiratory activity of the sperm after the original burst of activity upon dilution.

2. Gray's "theory of exponential decay" is not adequate to explain sperm metabolic activity, whereas the "theory of autointoxication" fits the activity of sperm cells suspended in seminal fluid.

3. The seminal fluid delays appreciably the onset of an irreversible destruction of part of the metabolic system.

4. Fertilization studies have led to the formulation of a tentative mechanism based on the adsorption of a protein substance and its removal from the surface of the sperm cell.

5. The proposed mechanism also explains adequately the results of the respiration experiments. Therefore, it is concluded that a seminal fluid factor, by its action while on the surface of the sperm, influences both the fertilizing capacity and the respiratory rate of spermatozoa.

6. Egg water added to a sperm suspension after the original dilution, causes a sharp decrease in the respiratory rate.

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